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The Behavior of Neuronal Cells on Tendon-Derived Collagen Sheets as Potential Substrates for Nerve Regeneration

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Abstract

Peripheral nervous system injuries result in a decreased quality of life, and generally require surgical intervention for repair. Currently, the gold standard of nerve autografting, based on the use of host tissue such as sensory nerves is suboptimal as it results in donor-site loss of function and requires a secondary surgery. Nerve guidance conduits fabricated from natural polymers such as collagen are a common alternative to bridge nerve defects. In the present work, tendon sections derived through a process named bioskiving were studied for their potential for use as a substrate to fabricate nerve guidance conduits. We show that cells such as rat Schwann cells adhere, proliferate, and align along the fibrous tendon substrate which has been shown to result in a more mature phenotype. Additionally we demonstrate that chick dorsal root ganglia explants cultured on the tendon grow to similar lengths compared to dorsal root ganglia cultured on collagen gels, but also grow in a more oriented manner on the tendon sections. These results show that tendon sections produced through bioskiving can support directional nerve growth and may be of use as a substrate for the fabrication of nerve guidance conduits.

1. Introduction

Peripheral nervous system (PNS) injuries result in a decreased quality of life due to factors such as neuropathic pain and oftentimes reduced mobility[1]. Complete nerve transections are a major problem, and on average there are 200,000 incidences every year that require surgical intervention in the United States alone[2]. These peripheral nerve injuries are commonly the result of trauma, such as automobile accidents, burns, explosive injuries, and can be a complication of some surgical procedures. As such, there exists a large clinical need for suitable nerve guidance products for peripheral nerve repair. When the damage is

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small, the two ends of the nerve stump can often be sutured together end-to-end in order to induce repair. However, this is only possible when the damaged nerve segment is less than several millimeters as tension has been shown to increase scarring and impair regeneration[3–5]. When this is not possible there are a number of other options available, including the gold standard of replacing the damaged portion with a nerve autograft. A limitation of this approach is loss of function at the donor site, while the repaired portion of nerve may not achieve complete functional restoration.

Over the past decade a number of options have emerged for peripheral nerve repair including nerve guidance conduits (NGC). The goal of a NGC is to provide a pathway along which two ends of a sutured nerve stumps can regrow towards each other. This regrowth involves several stages including fluid infiltration, formation of a fibrin matrix and migration of a variety of cell types including Schwann cells[6]. These events can be influenced greatly by the properties of the NGC, such as permeability, stiffness, and degradation rate [7].

A number of nerve guidance conduits are currently available on market including those comprised of synthetic polymers such as poly(lactide-co-glycolic acid) (PLGA), and polycaprolactone (PCL) [6], as well as natural materials such as animal extracted collagen[8,9] and silk [7,10,11]. Each of these NGCs have differing properties with advantages and disadvantages; a thorough review of FDA-approved NGCs can be found in Kehoe et al.[6].

Many nerve repair products in development make use of guidance strategies such as chemical or physical structures that serve to preferentially induce directional growth. The incorporation of growth factors into these structures generally functions to induce increased proliferation and provide a chemokine gradient along which the neuronal cells will grow. Physical features such as channels and grooves are similarly incorporated in order to provide nanotopographical cues which also guide directional growth along their length[12].

These physical structures are often incorporated into both synthetic and natural polymer-based nerve conduits through processing methods such as electrospinning and gel-casting. Electrospinning can produce nano-scale fibers of uniform dimensions that have been shown to guide cell growth[13,14], however this process has been shown to be more difficult with some materials, such as collagen, than synthetic polymers due to the water-insoluble nature of the collagen[15]. Additionally, the collagen fibers produced via this method do not recapitulate the complete native triple-helix and characteristic strength of native collagen fibers[16–18]. Other methods of incorporating aligned structures include extrusion[3], micropatterning using traditional photolithography techniques[19,20], and gel-casting/freeze drying[21]. One additional factor that must be considered when fabricating conduits out of ECM, such as collagen is that the material may shrink in vivo. This may be a result of a number of in vivo processes [22,23] such as crosslinking, or degradation by collagenases, but must be considered as it has the potential to place unwanted tension on the nerve stumps.

Our lab has recently demonstrated a technique, that allows us to create both flat and tubular scaffolds out of tendon-derived collagen that maintains the native collagen fiber structure[24,25]. This is in contrast to more common methods of producing collagen scaffolds that often involve enzymatic or acidic solubilization of the collagen, followed by reformation of a collagen gel into the desired structure. Our fabrication process (Figure 1) involves taking a block of tendon (such as bovine Achilles tendon), decellularizing the tissue in a sodium dodecyl sulfate solution, and then sectioning the block into thin sheets (5–100µm thick) using a microtome. These sheets can then be wrapped around rods of various diameters and crosslinked to form tubular conduits (Figure 2A,B). These conduits can also

be constructed with luminal fillers as we have previously shown, such as multiple smaller conduits within the primary NGC[24] or folded sheets of the tendon, in order to provide a three-dimensional surface for nerve regeneration. The benefit of this approach to fabricating collagen conduits is that it maintains the native structure of the collagen, as evidenced by the 67nm banding pattern (Figure 2C), as well as the native proteoglycans such as decorin[26], that may be beneficial in promoting axonal growth[27,28].

The goal of the present study was to evaluate the suitability of collagen sections created through our fabrication process for neuronal applications such as NGCs. Aligned growth, proliferation and adherence of various neural-related cell types were assessed.

2. Materials and Methods

2.1 Section Fabrication

Tendon sections were fabricated as previously described [24]. Briefly, we decellularized bovine Achilles tendon (Blood Farm, Groton, MA) in a 1% w/v sodium dodecyl sulfate (SDS) (Sigma, St. Louis, MO) solution for 48h and rinsed the tendon overnight to remove any residual SDS. The sections were frozen at -20°C and sectioned at $50\mu\text{m}$ thick using a cryomicrotome. The sections were placed on poly-lysine coated microscope slides (Polysine, Thermo Scientific, Billerica, MA) and then rinsed in deionized H_2O (diH_2O) for one hour to remove any of the embedding material. For adhesion and proliferation assays, the tendon sections were placed on 22mm diameter glass coverslips (Electron Microscopy Sciences, Hattisfield, PA). All samples were sterilized by soaking in 70% ethanol, rinsing 3x with PBS, followed by 1h of UV irradiation. Then, for specified experiments tendon samples, collagen gel-coated wells, or tissue culture poly styrene (TCPS) wells were coated in poly-D-lysine (PDL) (MW 70,000–150,000, Sigma) by filling the well with a 0.01mg/ml solution overnight and rinsing with phosphate buffered saline (PBS).

2.2 Collagen Sample Preparation

For adhesion, proliferation and DRG experiments collagen gel samples were prepared using Culturex 3-D Culture Matrix TM Rat Collagen I (Trevigen, Gaithersburg, MD). The gel was prepared in a 12-well tissue culture plate following the manufacturer's directions. For each plate, 250 μl of a 1mg/ml neutralized collagen gel was pipetted into each well, and the plate placed in a 37°C humidified incubator for 1 hour. Each well was then rinsed three times with PBS and either used as-is or coated with PDL as described above.

2.3 Scanning Electron Microscopy

The $50\mu\text{m}$ tendon samples were produced via sectioning and dehydrated in graded ethanol (10%, 20%, 50%, 95%, 100% v/v). The samples were sputter coated with Pt-Pd using a Cressington 208HR sputter coater (Watford, England) and imaged using at 6.0kV in a Zeiss FESEM Ultra55 (Oberkochen, Germany).

2.4 Schwann Cell Culture

Rat Schwann cells (CRL-2768, ATCC, Manassas, VA) were maintained in Dulbecco's Modified Eagles Medium (DMEM) (Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS) and Penicillin-Streptomycin (10,000 units Penicillin, 10,000 μg Streptomycin/mL) (Life Technologies) in a 37°C humidified incubator at 5% CO_2 . Cell morphology and alignment were observed via immunofluorescence by fixing the cells in 10% neutral buffered formalin (Sigma) for 10 minutes and then permeabilized with 0.1% (v/v) Triton X-100 (Sigma) for 5 minutes. After blocking with 1% (w/v) bovine serum albumin solution for 30min, the cells were stained

with rhodamine-phalloidin (Sigma) for 30min, counterstained the cell nuclei with 4',6-diamidino-2-phenylindole (DAPI) and observed via fluorescence microscopy.

2.5 Schwann Cell Adhesion Assay

For adhesion determination, Schwann cells were plated onto the samples (tendon, PDL-tendon, collagen, collagen-PDL, TCPS and PDL TCPS) in 12 well plates at a concentration of 100,000 cells in 50 μ l per well. Each substrate and time point was assayed in quadruplicate using cells of the same passage number. At a given time (0.5h, 1h or 2h) the wells were rinsed gently with PBS and aspirated to remove any non-adherent cells. To each well, 1ml of media was added with 100 μ L alamarBlue stock solution (Life Technologies). We then incubated the cells for 5 hour at 37°C. Following incubation, aliquots of the media were transferred to a black 96 well plated and the fluorescence determined using a microplate reader (excitation: λ =550nm, emission: λ =590nm). The results are presented as relative fluorescence units (RFU) normalized to background fluorescence.

2.6 Schwann Cell Proliferation Assay

For proliferative determination, Schwann cells were plated onto the samples (tendon, PDL-tendon, collagen, collagen-PDL, TCPS and PDL TCPS) in 12 well plates at a concentration of 100,000 cells in 50 μ L per well. After one hour the wells were rinsed with PBS to remove any non-adherent cells that may have otherwise adhered to the tissue culture plate, rather than the tendon substrates as desired. Media was then added and the remaining cells were monitored at time points of 1, 4, 7 and 10 days following seeding, via an alamarBlue assay as described above, with a 3 hour incubation period. Between assays the alamarBlue solution was removed and cells cultured in media. Each substrate was assayed in quadruplicate, with the same samples being used for each time point measurement.

2.7 Chick DRG Isolation, Culture and Staining

Chick dorsal root ganglia (DRG) were isolated from chicken eggs at development day E8. Immediately following isolation we seeded the chick DRG onto PDL-coated tendon samples, PDL-coated collagen gel and PDL-coated TCPS, and incubated them in DMEM supplemented with 2% FBS and 10ng/ml neurotrophin-3 (NT-3, Sigma). The DRG were cultured for 3, 5 or 7 days and then fixed and immunostained to determine morphology. We fixed the chick DRG in 4% (w/v) paraformaldehyde (Sigma) for 15 minutes and then permeabilized them with 0.1% (v/v) Triton X-100 (Sigma) for 5 minutes. After blocking with 2% (v/v) goat serum solution for 45 minutes, we incubated the cells with primary antibodies at 4°C overnight. The following antibodies were used for the staining: primary anti- β -tubulin-III (T2200, Sigma) (1:250) and secondary antibodies Alexa Flour 488 Goat Anti-Rabbit IgG (Life Technologies) (1:500). After washing with PBS, we added secondary antibodies and then incubated for 45min covered, followed by 3 washes with PBS for 15min each.

2.8 DRG Analysis

DRG outgrowth and orientation were analyzed with modification of the technique described by Richardson et al. [29–31]. Briefly, the perimeter of the DRG neurites was traced, and an ellipse fit to the perimeter using ImageJ. The dimensions of the major and minor axes were recorded and the value of the major axes reported as the length, while the aspect ratio = $\text{length}_{\text{major-axis}} / \text{length}_{\text{minor-axis}}$

2.9 Statistical Analysis

Data were analyzed for significance with IBM SPSS software, using a one-way ANOVA with *post hoc* Tukey's testing for each time point in the above assays. The α -value was set to 0.05 and results determined to be significant when $p < 0.05$.

3. Results

3.1 Schwann Cell Adhesion and Proliferation

Cells were seeded onto tendon sections, collagen and TCPS wells along with each substrate coated with PDL. The adhesion was measured after cells had incubated on the substrate for 0.5 hours, 1 hour and 2 hours (Figure 3A). At all time points, Schwann cells displayed the greatest adhesion on the PDL-coated TCPS while uncoated tendon and collagen displayed the least cell adhesion at 0.5 hours. The addition of PDL served to restore the adhesion at both time points when compared to both TCPS and PDL TCPS. After 2h hours of incubation, collagen showed greater adhesion compared to tendon, however at no time point was there any statistically significant difference between PDL-coated tendon and PDL coated collagen. In the proliferation assays (Figure 3B), a 1 hour incubation time was used prior to washing non-adherent cells and adding media to prevent non-adherent cells from being washed off of the tendon substrates. One day following cell seeding, no significant difference was seen between any conditions, however for the rest of the days the PDL coated tendon and TCPS had a greater number of cells, indicating increased proliferation. On day 10, PDL tendon and PDL TCPS had a greater number of cells than all other substrates, while the tendon and TCPS had a greater number of cells than the PDL and the PDL-coated collagen. A more detailed display of the *post hoc* analysis and statistical significance at each time point of the proliferation assay can be found in Supplementary Figure 1.

3.2 Aligned Growth of Schwann Cells

Differing morphologies were seen when Schwann cells were cultured on TCPS, collagen, and tendon. Cells on TCPS and Collagen displayed more spreading with no particular orientation of the actin cytoskeleton (Figure 4A,B), while cells cultured on tendon displayed a more condensed and elongated morphology with a distinct orientation following the direction of the tendon's collagen fibers (Figure 4C). No obvious differences in morphology were seen between each substrate and the equivalent substrate coated with PDL (Figure 4D–F). Any differences in perceived cell density in Figure 4 images are a result of attempting to obtain clear, representative images of the cytoskeleton rather than a reflection of actual cell density.

3.3 DRG Growth and Extension

Chick DRG at stage E8 adhere to TCPS, collagen and tendon coated with PDL. By day 3, neurite extensions can be seen on all substrates, with DRG on TCPS and collagen displaying round extension of neurites (Figure 5A,B), while DRG on tendon appear to have extension in the direction of the tendon fibers (Figure 5C). This pattern continues for DRG cultured for 5 days (Figure 5D–F) as well as 7 days (Figure 5G–I). As DRG neurite extensions grow more dense after being cultured for longer time periods on the tendon substrate, neurites appear to extend radially directly surrounding the main DRG body. Once these neurites reach past the direct branching they begin following the tendon fibers.

On all substrates DRG extend at similar rates (Figure 6A), reaching total lengths of 2.93 ± 0.06 mm, 3.67 ± 0.29 mm and 4.13 ± 0.31 mm by day 7 for TCPS, collagen, and tendon substrates, respectively. Measurement of the aspect ratio (Figure 6B) of DRG growth on the substrates shows that DRG grow in one direction preferentially on the tendon substrate

compared to the other substrates, at all of the time points observed. The total area of DRG growth (Supplementary Figure 2) was less on the tendon substrate compared to the collagen gel, however this does not reflect density of the neurites, which was not quantified in these experiments. N=4, 7, 8 for TCPS, collagen, and tendon, respectively, at each time point.

4. Discussion

The objective of this project was to evaluate the suitability of tendon-derived scaffolds produced via our fabrication process for neuronal cell growth, with the ultimate goal of use as a NGC. Collagen in many forms has proven to be a suitable biomaterial for a number of biomedical applications[32–34], and as such we reasoned that the tendon-derived collagen sheets would also be useful as an NGC. The procedure shown in Figure 1A can be used to produce collagen NGC of varying lengths and diameters that can be handled easily in both the dry and hydrated states. These NGCs consist of several layers of alternating fiber orientations, where the interior layers are comprised of collagen nanofibers that run parallel to the length of the conduit and subsequent layers alternate proceeding outwards. This fiber orientation ensures that cells will be in contact with fibers in the desired orientation while the alternating fibers provide strength and allow for easy suturing as they prevent pull-out of the sutures more than an NGC composed entirely of aligned fibers. Figure 2A demonstrates the general appearance of a tubular conduit while Figure 2B shows the cross-sectional area of a portion of the conduit.

The growth of Schwann cells, along with their adhesion and proliferation are an important as they are an integral part of the peripheral nerve repair process [12]. At several of the time periods observed, collagen and tendon substrates had reduced adhesion compared to the synthetic TCPS. The addition of positively charged PDL was able to increase adhesion of all substrates at each of the time points, including increasing the adhesion of Schwann cells on tendon and collagen to comparable levels with TCPS (Figure 3A). This is most likely a result of the positively charged PDL increasing the adhesion of the Schwann cells through a charge interaction [35,36].

Measurement of the proliferation of Schwann cells (Figure 3B) also showed that growth on PDL coated tendon as well as PDL coated TCPS exceeded that of the uncoated substrates at days 7 and 10. The increased proliferation may be a result of both increased initial adhesion and a result of the properties of PDL itself [36]. In this experiment, collagen displayed significantly lower proliferation than the other substrates, and the addition of PDL did not appear to increase the proliferation. We hypothesize that this may be a result of the collagen gel hydrating over time, resulting in the cells losing grip of where they're adherent to the substrate, whereas the native collagen molecule in tendon may not hydrate and disperse as easily. This increased growth of Schwann cells on the tendon is an important consideration for in vivo applications as increased proliferation of Schwann cells has been shown to influence the viability of NGC [37].

In addition to adhesion and proliferation, the alignment and morphology of the Schwann cells on each substrate was considered (Figure 4). Cells cultured on TCPS or collagen appeared to have no defined orientation, and spread randomly on the substrate (Figure 4A,B,D,E). Tendon, however, causes Schwann cells to align in a direction parallel to the collagen fibers in a very defined manner (Figure 4C,F). Alignment of Schwann cells has been shown to upregulate markers of myelination and downregulate neurotrophin and neurotrophic receptors associated with an immature Schwann cell phenotype [14]. Aligned Schwann cells, in the form of bands of Bünger, are also a crucial step during regrowth of axons in nerve repair following Wallerian degeneration[14,38].

Here, we have also demonstrated the growth of DRG on the different substrates. Growth of neurites from DRG on TCPS and collagen gels appear to extend radially from the DRG body while neurites extending from DRG on the tendon grow along the orientation of the tendon's collagen fibers (Figure 5). This organization of the neurite outgrowth as demonstrated by the DRG is important in regards to peripheral nerve regeneration as maintenance of functional organization in the repairing nerve is key to prevent wrong connections at the distal end of the damaged nerve [39,40]. No significant difference was seen in the length of growth (Figure 6A), between the tendon substrate, collagen substrate, or TCPS. However the greatly increased degree of alignment (Figure 6B) observed on the tendon may be beneficial for in vivo nerve repair and proper connection formation.

The radial growth, followed by the directional growth that can be seen in day 7 DRG may be explained by the complex nature of neurite outgrowth and nerve growth cone extension. As discussed by Maskery[41], this may be a result of differing adhesion or chemotactic cues. We hypothesize that neurites are initially growing on top of one another close to the DRG body and extend radially. Once they reach a sufficient distance from the DRG body, they come into contact with the tendon substrate and their direction of growth is influenced. The directional change caused by the tendon should also be noted, as growth cones have a tendency to grow in a uniformly straight path on flat surfaces[40,41].

DRG isolated from several species, such as chicken and rat embryos have been used to model nerve growth for various purposes, including guiding or directing oriented neurite growth [39,42]. Different methods of directing the growth of chick DRG have been used including micropatterning growth factors[43], and proteins[44] onto surfaces. Other experiments have utilized physical cues such as magnetically aligned collagen fibers[39], Schwann-cell-mimicing topography in poly(dimethylsiloxane) [29] or electrospun fibers [13,42]. The Schwann cell-patterned conduits and electrospun PLL conduits were able to achieve oriented DRG growth on synthetic polymers, indicating that the orientation observed on the tendon substrates can be a result strictly from physical cues rather than a result of the inherent property of the collagen. However, the growth of DRG on electrospun poly- ϵ -caprolactone (PCL) fibers compared to a blend of collagen and PCL resulted in greater alignment but decreased neurite length on the collagen-containing substrate[42]. This indicates that the alignment may also be influenced by some adhesion molecule or other binding site found on collagen.

5. Conclusions

The growth and alignment of Schwann cells and DRG along the tendon substrate show that the nanofibrous collagen found in tendon has the ability to direct neuronal cell growth. The proliferation of these cells has also been shown to be comparable to collagen gel, commonly used in many current FDA-approved nerve guidance conduit. Additionally, the added alignment that is a result of collagen fibers may result in a more mature Schwann cell phenotype and may promote the formation of the correct connections in re-growing axons, as shown by chick DRG growth. Overall, tendon sections produced through our fabrication process have potential for improving peripheral nerve regeneration if used as a nerve guidance conduit and will be further investigated through in vivo experimentation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Scheme showing fabrication and use potential of NGCs. **A)** Process use to fabricate the tendon sections, involving removing a block from the bulk tendon, sectioning the decellularized block and then wrapping multiple sheets around a tubular rod. **B)** Illustration of the directional alignment of DRG along the collagen fibers. **C)** Illustration of the multilayer tubular structures fabricated from the collagen sheets used as a NGC.

Figure 2.

A) Photograph of several tubular conduits of various lengths formed via bioskiving. **B)** Electron micrograph of the cross-section of a tubular conduit. **C)** Electron micrograph of aligned collagen fibrils in the tendon samples, showing the characteristic 67nm banding pattern of collagen.

Figure 3.

A) Adhesion of Schwann cells on tendon, collagen or TCPS, along with each substrate coated with PDL; Measured via AlamarBlue assay after cells are incubated on the surface for 0.5h, 1h and 2h before having non-adherent cells washed off. No difference is seen in adhesion after 1h of incubation. Statistical significant of $p < 0.05$ indicated by * and $p < 0.005$ by **. **B)** Proliferation rate (via AlamarBlue assay) of Schwann cells on the same culture surfaces measured at 1, 4, 7 and 10 days. An initial incubation period of 1h was used. Detailed statistical differences can be found in Supplementary Figure 1; PDL tendon and PDL TCPS are statistically greater than other substrates at days 7 and 10.

Figure 4.

Fluorescence images of Schwann cells cultured for 1 day on: **A)** TCPS, **B)** collagen gel, **C)** tendon, **D)** PDL-coated TCPS, **E)** PDL-coated collagen, **F)** PDL-coated tendon. Cells orient along collagen fibers in tendon with and without the PDL coating. No difference is seen in morphology with the PDL coating. Red is F-actin staining with rhodamine-phalloidin and blue is nuclei staining with DAPI.

Figure 5.

Representative fluorescence images of chick DRG isolated at E8 and cultured for 3, 5 or 7 days. DRG were cultured on PDL-coated TCPS (**A,D,G**), PDL-coated collagen gel (**B,E,H**), and PDL-coated tendon sections (**C,F,I**). DRG cultured on tendon sections extend neurites along the orientation of the collagen fibers in the tendon, while DRG cultured on TCPS and the collagen gel extend neurites radially. Green is anti- β -III tubulin staining.

Figure 6.

Growth of DRG after culture for 3, 5 or 7 days on TCPS, collagen and tendon, each with PDL. **A)** Length in mm of the longest direction of neurite growth, in a straight line through the DRG body. No significant difference between substrates at 3, 5 or 7 days. **B)** Aspect ratio of DRG growth indicating directional growth, where the aspect ratio is the length of the major axis divided by the length of the minor axis. DRG cultured on tendon shows much greater directional growth at all points of time. Statistical significance of $p < 0.005$ indicated by ** and $p < 0.0005$ by ***.